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14. ABSTRACT We have proposed that prostate cancers adapt to androgen deprivation therapy by increasing their synthesis of potent androgens from available weak adrenal androgens (and possibly from endogenous precursors), and that AKR1C3 is a key enzyme in this process. Our objectives are to test these hypotheses using cell line and xenograft models (Aim 1) and by measuring androgen and androgen metabolite levels in patients who progress to CRPC (Aim 2). The progress reported here strongly supports the conclusion that a mechanism for tumor progression after androgen deprivation is increased intratumoral androgen synthesis. Further xenograft studies will focus on the VCaP model using additional drugs and shRNA approaches to inhibit key enzymes in androgen synthesis. Studies in clinical material will focus on the analysis of additional samples, and on correlating effects on androgen levels with clinical responses.

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INTRODUCTION

The majority of prostate cancers (PCa) are androgen dependent, and androgen deprivation therapy (ADT) remains as the standard treatment for non-organ confined disease. Unfortunately, patients treated with ADT invariably relapse with rapidly progressive systemic PCa, which has been termed hormone refractory, androgen independent, or castration resistant prostate cancer (CRPC). Significantly, the androgen receptor (AR) is highly expressed in most cases of CRPC and appears to be transcriptionally active, but the molecular events mediating the progression to CRPC and apparent reactivation of AR transcriptional activity remain to be defined. Our previous data indicate that increased intratumoral production of testosterone from precursors (adrenal androgens and possibly endogenous sterols) contributes to the reactivation of AR transcriptional activity in CRPC. We propose that tumors adapt to androgen deprivation therapy by increasing their synthesis of potent androgens from available weak adrenal androgens (and possibly from endogenous precursors), and that AKR1C3 is a key enzyme in this process. Our objectives are to test these hypotheses using cell line and xenograft models (Aim 1) and by measuring androgen and androgen metabolite levels in patients who progress to CRPC (Aim 2). If the intracellular production of potent androgens from adrenal or endogenous precursors is indeed a mechanism for progression to CRPC, then this pathway would become a new therapeutic target.

BODY

Aim 1. Test the hypothesis that increased expression of *AKR1C3* enhances conversion of androstenedione to testosterone and stimulates tumor growth after androgen deprivation therapy in cell line and xenograft models (months 1-36)

In last year's report we outlined our preliminary data using the VCaP cell line and xenograft model to assess the role of AKR1C3 in driving androgen synthesis and tumor growth after androgen deprivation. These studies have been completed during the current year and a manuscript detailing the data is now in press and included in the Appendix (Cai et al., 2009).

Having established that AKR1C3 and other enzymes mediating androgen synthesis are increased in this model of CRPC, our recent efforts have focused on determining whether blocking this synthesis will suppress tumor growth. The two general approaches are pharmacological and genetic (shRNA knockdown of key enzymes). Specific AKR1C3 inhibitors are not yet available, but indomethicin inhibits AKR1C3 at clinically achievable levels. Therefore, we have assessed the efficacy of indomethicin in the VCaP cell line and xenograft model of CRPC. As shown in figure 1A, indomethicin could markedly suppress basal and androstenedione stimulated PSA expression in VCaP cells (Fig. 1A, left panel), but was less effective in LNCaP cells (Fig. 1A, middle panel). Significantly, LNCaP expresses a mutant AR (T877A) that can be stimulated by androstenedione, suggesting that much of the stimulatory effect of androstenedione is direct and not mediated by AKR1C3 mediated conversion to testosterone. Consistent with this conclusion, LNCaP cells express very low levels of AKR1C3 relative to VCaP cells (Fig. 1B). Ketoconazole, an inhibitor of an upstream step in androgen synthesis (CYP17A1), decreases basal PSA expression in VCaP cells (in steroid depleted medium), suggesting that these cells synthesize low levels of androgens de novo from cholesterol (Fig. 1A, right panel). However, as expected, ketoconazole does not prevent block PSA expression in response to androstenedione. Taken

together, these data strongly support the conclusion that AKR1C3 can mediate AR reactivation when there is androstenedione available for conversion to testosterone.

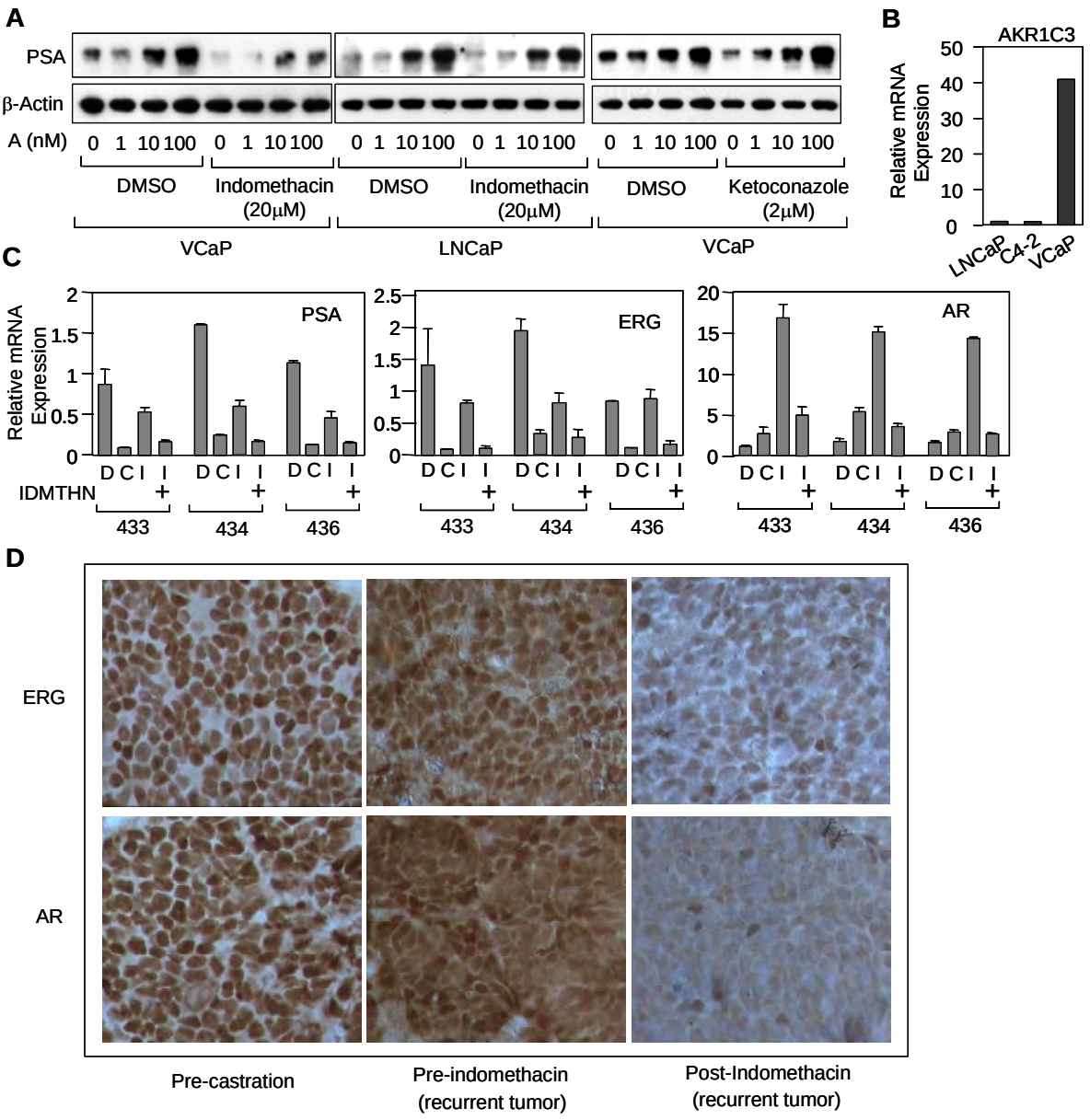

Fig. 1. A, VCaP and LNCaP cells in steroid depleted medium were treated for 24hr with the indicated drugs (A, androstenedione), and equal amounts of total protein were then immunoblotted. B, expression of AKR1C3 was assessed by real time RT-PCR. C, VCaP xenografts in three mice were biopsied prior to castration (androgen dependent, D), at 4 days after castration (C), and at the time of recurrence (androgen independent, I) prior to or after 10 days of indomethicin treatment (added to drinking water). Expression of PSA, ERG (from TMPRSS2:ERG fusion gene) and AR were assessed by real time RT-PCR. C, immunohistochemistry in tumors from a representative mouse.

We next carried out studies of indomethicin in vivo in CRPC VCaP xenografts. We first implanted VCaP cells subcutaneously into a series of immunodeficient (SCID) mice. When tumors became palpable, the mice were castrated and then observed until tumors regrew. At that time the mice were treated with indomethicin, which was added to the drinking water. Figure 1C shows an analysis of biopsies of tumors from three mice taken before castration (androgen dependent, D), at 4 days after castration (C), and at the time of recurrence (androgen independent, I) prior to or after 10 days of indomethicin treatment. As expected, PSA and TMPRSS2:ERG mRNA levels declined markedly after castration, and partially recovered in the recurrent androgen independent tumors (Fig. 1C). Also as shown previously, AR mRNA levels were increased in after castration and were further increased in the recurrent tumors. Significantly, indomethicin treatment markedly decreased PSA and TMPRSS2:ERG mRNA in the recurrent tumors to levels that were comparable to those after castration, consistent with a critical role for AKR1C3 in mediating testosterone production from circulating androstenedione in these tumors. Direct measurements of testosterone in these tumors is currently underway to further support these conclusions.

Immunohistochemistry pre and post indomethicin confirms the marked effects of the drug on expression of AR and the AR regulated TMPRSS2:ERG (Fig. 1D). It should be noted that the suppression of AR mRNA expression and AR protein by indomethicin were not expected, but may clearly contribute to the response. Further studies, including efforts to understand the molecular basis for decreased AR expression, are underway. In addition to indomethicin, we have recently obtained the CYP17A1 inhibitor abiraterone (via an MTA with Cougar Pharmaceuticals) and will soon be able to launch in vivo studies using this agent to block androgen synthesis. Efforts to generate xenografts with tetracycline regulated AKR1C3 shRNA expression are also in progress.

Aim 2. Test the hypothesis that serum levels of androgen metabolites are increased in patients with progression to androgen independence (months 1-36)

We have collected and analyzed serum samples from a 57 men with CRPC who were starting therapy on a clinical trial of ketoconazole, hydrocortisone, and dutasteride. Table 1 shows the results of these hormone measurements, and also shows how these hormone levels changed over time with treatment. All levels dropped in response to the therapy, but there were no clear correlations between clinical responses and hormone levels prior to therapy or the magnitude of decline after therapy. In patients with clinical responses there was no clear increase at the time of relapse.

A manuscript further detailing these hormone studies and clinical results has been submitted for publication, and is included in the Appendix (Taplin et al. submitted for publication). Importantly, the high response rate and prolonged time to progression indicates that this therapy is effective, and further clinical trials are being developed. In addition to these serum samples, we have added to our collection of serum samples from men in remission after ADT, and hormone measurements in these are pending.

Table 1. Hormone levels over time among all patients

	Baseline	Month 1		Month 2		Month 3	
Hormone	Value	Value	Percent change from baseline	Value	Percent change from baseline	Value	Percent change from baseline
N patients	N=41	N=33	N=30	N=28	N=24	N=28	N=24
Androstene-dione (ng/ml)	0.84 (0.61, 1.10)	0.22 (0.20, 0.36)	-56% (-75%, -43%)	0.27 (0.20, 0.32)	-68% (-74%, -49%)	0.25 (0.20, 0.31)	-58% (-77%, -49%)
DHEA-S (ng/ml)	599 (340, 121)	65 (36, 125)	-89% (-94%, -70%)	65 (37, 106)	-92% (-94%, -77%)	65 (35, 100)	-90% (-94%, -81%)
Testosterone (ng/ml)	0.37 (0.26, 0.47)	0.13 (0.10, 0.15)	-66% (-72%, -55%)	0.11 (0.9, 0.14)	-68% (-73%, -54%)	0.12 (0.10, 0.19)	-64% (-72%, -45%)
DHT[1] (pg/ml)	2.6 (<2.0, 3.8)	<2.0 (<2.0, <2.0)					

Values are median and interquartile range (25^{th}, 75^{th} percentiles).
[1]Note: For DHT, n=33 at baseline, n=32 at month 1 and n=29 for percent change.

KEY RESEARCH ACCOMPLISHMENTS

- Established VCaP as a xenograft model for increased androgen synthesis in prostate cancer progression
- Demonstrated efficacy of an AKR1C3 inhibitor in this model
- Expanded collection of serum samples for hormone measurements
- Assessed hormone levels in a clinical trial of androgen synthesis inhibitors in CRPC

REPORTABLE OUTCOMES

Cai,C., Wang, H., Xu, Y., Chen, S., and Balk, S.P. (2009). Reactivation of Androgen Receptor Regulated TMPRSS2:ERG Gene Expression in Castration Resistant Prostate Cancer. Cancer Res., in press.

CONCLUSION

The recent work strongly supports the conclusion that a mechanism for tumor progression after androgen deprivation is increased intratumoral androgen synthesis. Further xenograft studies will focus on the VCaP model using additional drugs and shRNA approaches to inhibit key enzymes in androgen synthesis. Studies in clinical material will focus on the analysis of additional samples, and on correlating effects on androgen levels with clinical responses.

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Cai,C., Wang, H., Xu, Y., Chen, S., and Balk, S.P. (2009). Reactivation of Androgen Receptor Regulated TMPRSS2:ERG Gene Expression in Castration Resistant Prostate Cancer. Cancer Res., in press.

APPENDICES

One manuscript in press.
One manuscript submitted for publication.

Reactivation of Androgen Receptor–Regulated TMPRSS2:ERG Gene Expression in Castration-Resistant Prostate Cancer

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Abstract

It seems clear that androgen receptor (AR)–regulated expression of the *TMPRSS2:ERG* fusion gene plays an early role in prostate cancer (PC) development or progression, but the extent to which *TMPRSS2:ERG* is down-regulated in response to androgen deprivation therapy (ADT) and whether AR reactivates *TMPRSS2:ERG* expression in castration-resistant PC (CRPC) have not been determined. We show that ERG message levels in *TMPRSS2:ERG* fusion-positive CRPC are comparable with the levels in fusion gene–positive primary PC, consistent with the conclusion that the *TMPRSS2:ERG* expression is reactivated by AR in CRPC. To further assess whether *TMPRSS2:ERG* expression is initially down-regulated in response to ADT, we examined VCaP cells, which express the *TMPRSS2:ERG* fusion gene, and xenografts. ERG message and protein rapidly declined in response to removal of androgen *in vitro* and castration *in vivo*. Moreover, as observed in the clinical samples, ERG expression was fully restored in the VCaP xenografts that relapsed after castration, coincident with AR reactivation. AR reactivation in the relapsed xenografts was also associated with marked increases in mRNA encoding AR and androgen synthetic enzymes. These results show that expression of *TMPRSS2:ERG*, similarly to other AR-regulated genes, is restored in CRPC and may contribute to tumor progression. [Cancer Res 2009;69(15):OF1–6]

Introduction

A major breakthrough in prostate cancer (PC) was identification of recurrent fusions between androgen-regulated *TMPRSS2* and Ets transcription factor genes (primarily *ERG*), placing the *Ets* genes under androgen-stimulated regulation of *TMPRSS2* (1). Remarkably, this fusion is in preneoplastic lesions and ~50% of primary PC, consistent with an early role in tumor development (1–6). The standard treatment for locally recurrent/metastatic PC is androgen deprivation therapy (ADT), but patients invariably relapse with more aggressive tumors termed castration-resistant PC (CRPC). Significantly, androgen receptor (AR) is expressed at high levels in CRPC, as are multiple AR-regulated genes, indicating that AR transcriptional activity is at least partially reactivated (7, 8). Mechanisms contributing to this reactivation include increased intratumoral androgen accumulation/synthesis (8–12), AR overexpression, AR mutations (in AR antagonist–treated patients), and activation of kinase pathways that enhance AR activity.

TMPRSS2 is decreased in response to ADT (13), and it is presumed that *TMPRSS2:ERG* expression would also be decreased, which may contribute to responses, but this has not been shown directly in patients. The extent to which the *TMPRSS2:ERG* gene is expressed in CRPC and contributes to relapse is also unclear. One study of CRPC with the *TMPRSS2:ERG* gene found that it was not expressed, but this was in atypical AR-negative tumors (6). In contrast, the initial identification of fusion gene transcripts included CRPC tumors, although these were a small subset of outliers expressing very high ERG message levels (1). Therefore, to determine the extent to which *TMPRSS2:ERG* gene expression is reactivated in CRPC, we examined ERG expression in *TMPRSS2:ERG* fusion-positive primary androgen-dependent PC and CRPC clinical samples and in VCaP xenografts (14) before and after castration.

Materials and Methods

Cell culture and xenografts. Cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS). For DHT treatment, cells were first grown to 50% to 60% confluence in 5% charcoal/dextran-stripped FBS (CSS) medium for 3 d. VCaP xenografts were established in the flanks of male severe combined immunodeficient mice by injecting ~2 million cells in 50% Matrigel. When tumors reached ~1 cm, biopsies were obtained and the mice were castrated. Additional biopsies were obtained 4 d after castration, and the tumors were harvested at relapse. Frozen sections confirmed that samples contained predominantly nonnecrotic tumor.

Reverse transcription-PCR and immunoblotting. Real-time reverse transcription-PCR (RT-PCR) used 50 ng RNA, and the results were normalized by coamplification of 18S RNA (see Supplementary Data). Blots were incubated with anti-ERG (1:1,000, polyclonal; Santa Cruz Biotechnology), anti–prostate-specific cancer (PSA; 1:3,000, polyclonal; BioDesign), anti-AR (1:2,000, polyclonal; Upstate), anti-pAR(Ser^{81}) (1:1,000, polyclonal; Upstate), or anti-actin (1:5,000, monoclonal; Abcam) and then with secondary antibodies (Promega).

Immunohistochemistry. Paraffin sections were boiled for 30 min in 10 mmol/L citrate buffer (pH 6.2) and blocked using 5% goat serum and avidin blocking solution (Vector). Primary antibodies, anti-AR (1:50) or anti-ERG (1:200), were added overnight at 4°C followed by biotinylated goat anti-rabbit antibody (1:400) and streptavidin-horseradish peroxidase (1:400; Vector). Slides were developed with 3,3′-diaminobenzidine and counterstained with hematoxylin.

Results and Discussion

ERG is expressed at comparable levels in *TMPRSS2:ERG*-positive primary PC and CRPC. Using RT-PCR on RNA from previously described CRPC bone marrow metastases (8), we detected *TMPRSS2:ERG* transcripts (*TMPRSS2* exon 2-*ERG* exon 4) in 11 of 29 cases. Affymetrix oligonucleotide microarray data on these tumors versus a group of 27 microdissected primary PC (from the same study) were then examined for ERG expression.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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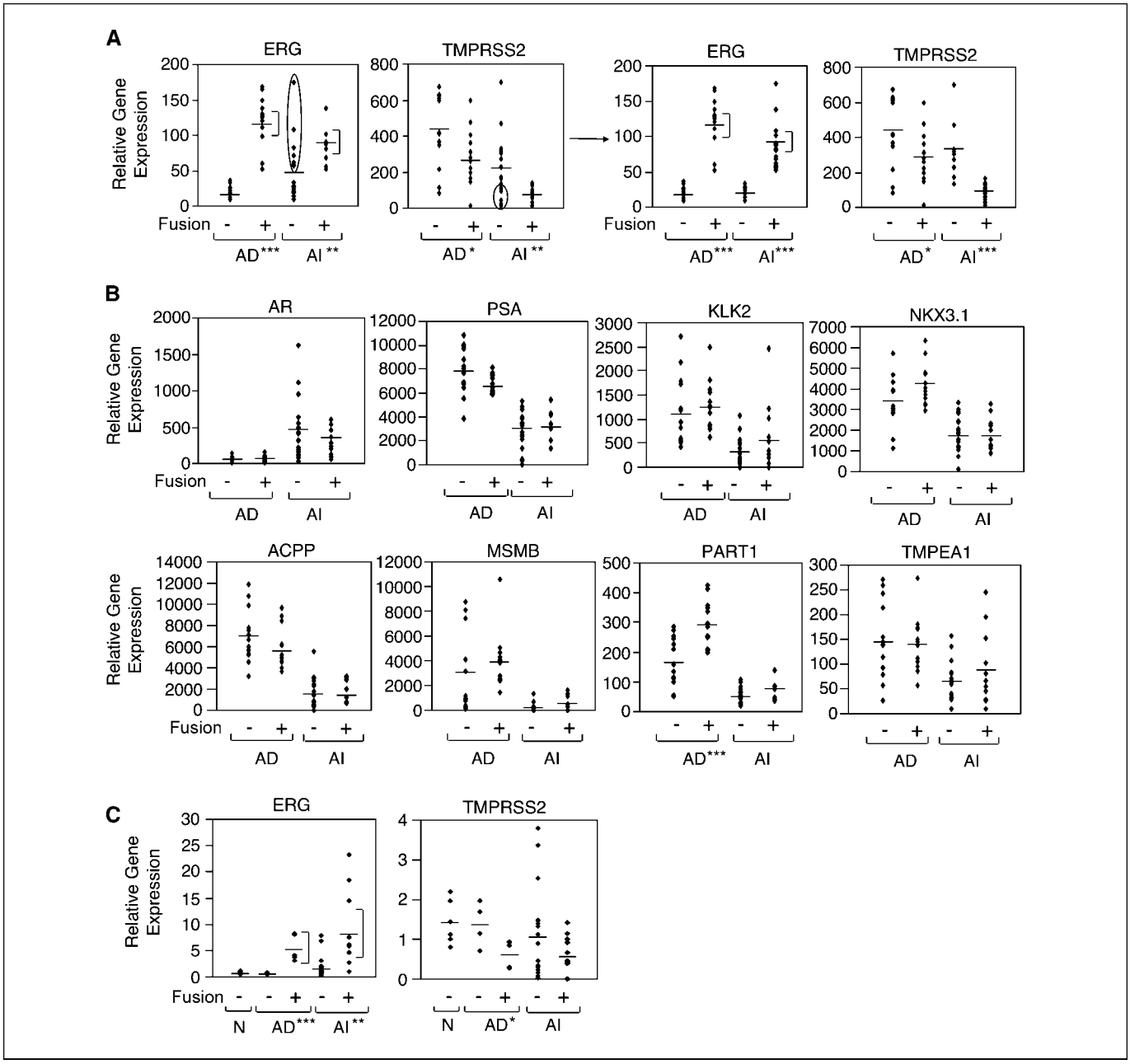

Figure 1. ERG expression in *TMPRSS2:ERG*-positive primary PC and CRPC. *A* and *B,* expression in fusion-negative versus fusion-positive primary PC [androgen dependent (*AD*)] and CRPC [androgen independent (*AI*)]. *C,* ERG and TMPRSS2 expression by RT-PCR in these 29 CRPC tumors (*AI*) versus another group of 10 untreated primary PC (*AD*) and 6 normal prostates (*N*). *Brackets,* 95% confidence intervals for fusion-positive tumors. *P* values for differences between fusion-negative and fusion-positive androgen-dependent or androgen-independent tumors are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Although the fusion status of the latter primary tumors was not known (RNA and tumor tissues were no longer available), ERG expression distinguished two nonoverlapping groups that presumably reflected fusion-negative (13 of 27) and fusion-positive tumors (14 of 27; Fig. 1*A, AD, left*).

The majority of the fusion-negative CRPC had low ERG levels that were comparable with the levels in the ERG low primary PC group (Fig. 1*A, AI, left*). However, ERG expression in six of the fusion-negative CRPC was higher (Fig. 1*A, circled*), suggesting *TMPRSS2:ERG* fusions that were not picked up by RT-PCR. These six samples also all had low TMPRSS2 mRNA levels, suggesting a *TMPRSS2:ERG* fusion and loss of TMPRSS2 expression from one allele (Fig. 1*A, left, circled*). The panels on the right of Fig. 1*A* show these six tumors reclassified as fusion positive, which would indicate that ERG is expressed at comparable low levels in the fusion-negative primary (androgen dependent) and castration-resistant (androgen independent) tumors. Importantly, independent of how these six tumors are classified, ERG expression in the fusion-positive CRPC was significantly higher than in the fusion-negative CRPC. Moreover, ERG expression in the fusion-positive androgen-dependent and androgen-independent tumors was comparable based on overlapping confidence intervals, with a

>95% probability that levels in androgen independent tumors are at least 50% of those in androgen-dependent tumors (Fig. 1*A*).

We reported previously that AR mRNA was consistently increased in CRPC and that multiple AR-regulated genes were highly expressed (8). As shown in Fig. 1*B*, expression of AR-regulated and androgen-regulated genes was similarly increased in the fusion-positive versus fusion-negative CRPC, consistent with comparable AR reactivation in these tumors. Interestingly, whereas expression of most AR-regulated genes was 2- to 3-fold lower in CRPC, ERG expression seemed to be more fully restored, suggesting that factors in addition to AR may be further enhancing *TMPRSS2:ERG* expression in CRPC.

To confirm these results, we examined ERG expression by real-time RT-PCR in the CRPC samples and in another independent small set of fusion-negative and fusion-positive primary PC. Significantly, this analysis also showed that ERG expression in the fusion-positive CRPC samples was increased ~4-fold compared with the fusion-negative CRPC and was comparable with expression in the fusion-positive primary PC (Fig. 1*C*). Taken together, these results show that the *TMPRSS2:ERG* fusion gene is expressed in CRPC at levels that are comparable with those in untreated primary PC, which presumably reflects at least in part reactivation of AR.

Expressions of *TMPRSS2:ERG* transcript and ERG protein are androgen stimulated in VCaP cells. Although the above data establish that *TMPRSS2:ERG* is comparably expressed in primary PC and CRPC, we have not yet been able to directly follow fusion gene expression *in vivo* in patients during ADT. Therefore, we next examined VCaP cells, which express AR and the common *TMPRSS2:ERG* fusion gene (14). Expression of an ~50 kDa protein, Q3
consistent with NH_2-terminal truncated ERG, could be induced rapidly and at low DHT levels (0.1 nmol/L) in VCaP but not fusion-negative LNCaP cells (Fig. 2*A*). ERG, PSA, and TMPRSS2 (from the F2
intact allele) mRNA were similarly induced in VCaP (Fig. 2*B*). Interestingly, induction was ~2-fold higher for *TMPRSS2* than *ERG*, suggesting that additional proteins may be increasing basal *TMPRSS2:ERG* expression. An AR antagonist, bicalutamide, suppressed DHT-stimulated expression of ERG (Fig. 2*C*). Taken together, these observations indicate that AR is similarly regulating both the wild-type *TMPRSS2* and the *TMPRSS2:ERG* fusion gene.

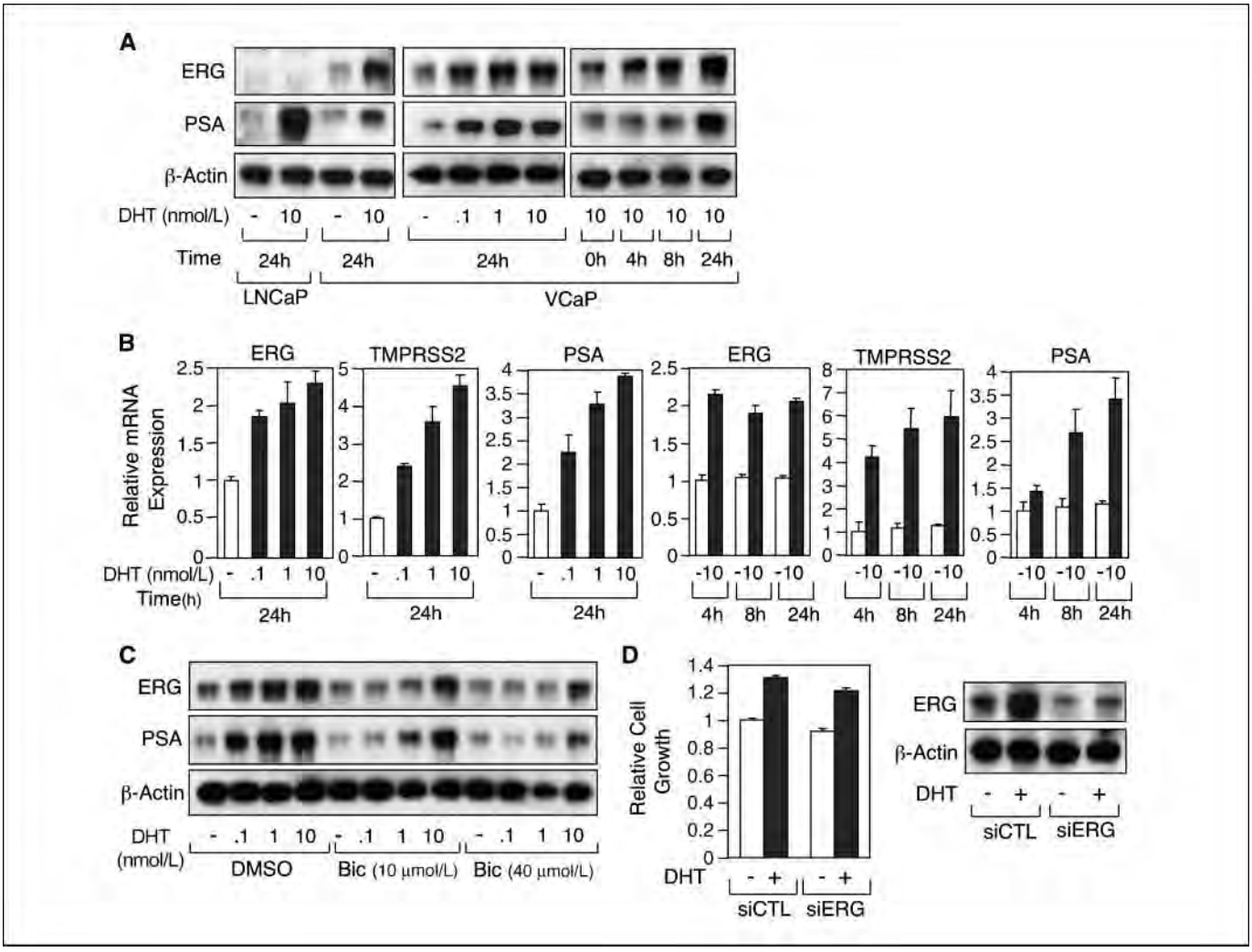

Figure 2. Androgen-regulated TMPRSS2:ERG expression in VCaP. *A,* cells in CSS medium were treated with DHT and immunoblotted. *B,* RT-PCR for ERG (exon 9/10), TMPRSS2 (exon 5/6), and PSA mRNA after DHT stimulation. *C,* cells in CSS medium treated with DHT and bicalutamide (*Bic*) for 24 h. *D,* cells in 5% CSS for 3 d were transfected with 10 nmol/L control or ERG siRNA (Dharmacon). DHT was added 8 h after transfection and cells were assayed for ERG protein and cell recovery [by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay] after 3 d (with comparable results at 5 d).

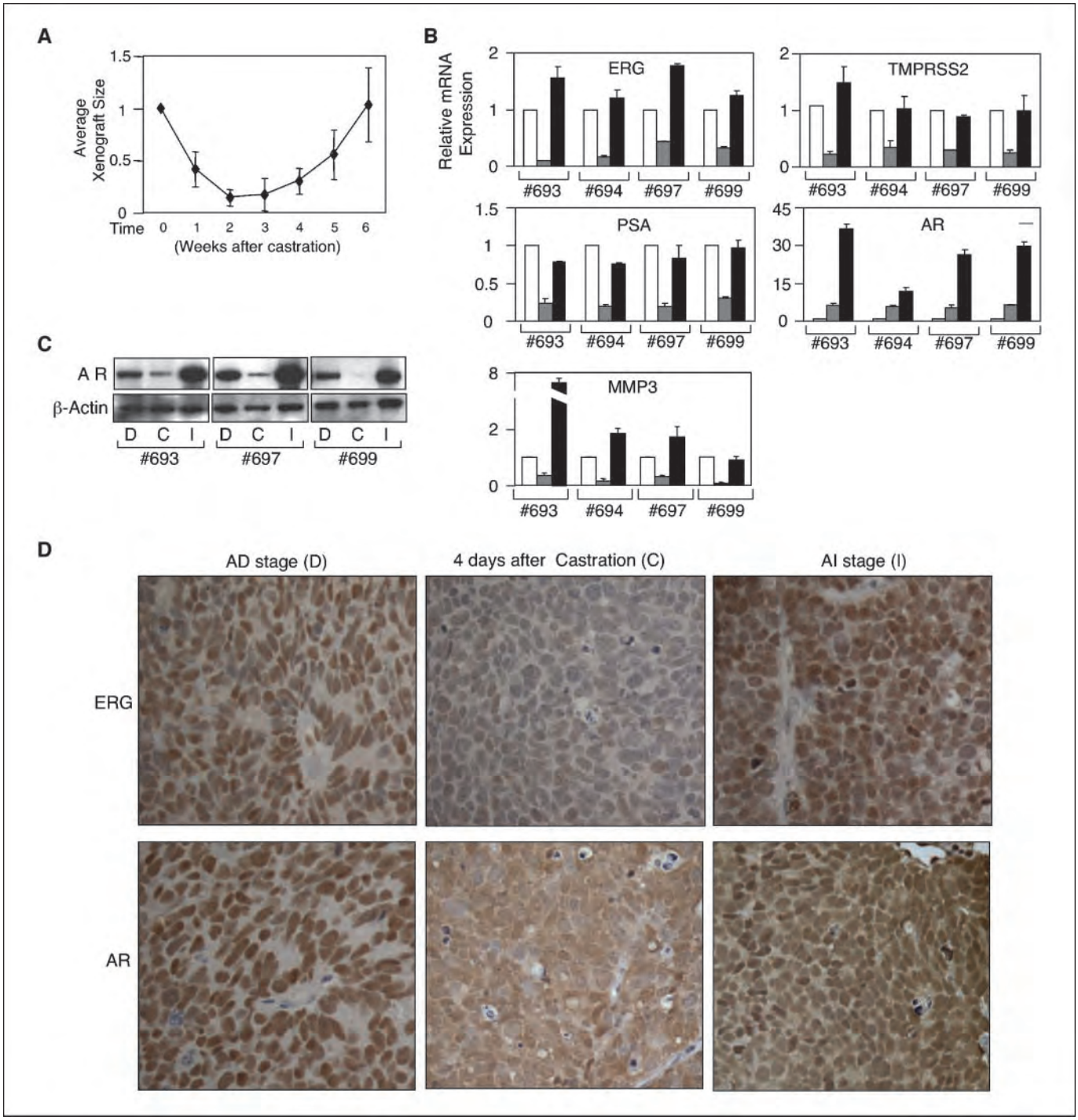

Figure 3. TMPRSS2:ERG expression in VCaP xenografts. *A,* average normalized xenograft size (±SD) at 1 to 6 wk after castration ($n = 6$). *B,* ERG, TMPRSS2, PSA, AR, and MMP3 mRNA in xenografts from four mice before castration (androgen dependent, *white columns*), 4 d after castration (*gray columns*), or at relapse (androgen independent, *black columns*). *C,* AR protein levels in xenografts before castration (*D*), 4 d after castration (*C*), and at relapse (*I*). *D,* immunohistochemistry for ERG and AR in representative xenograft.

Finally, using small interfering RNA (siRNA) to decrease ERG expression, we did not observe marked effects on cell growth in the presence or absence of DHT (Fig. 2*D*).

***TMPRSS2:ERG* expression in VCaP xenografts is ablated by castration and reactivated in relapsed tumors.** S.c. VCaP xenografts were biopsied before castration, at 4 days after castration, and at ~6 weeks when tumors were growing rapidly and reached ~1 cm (Fig. 3*A*). As expected, PSA mRNA was decreased at 4 days and restored in the relapsed tumors (Fig. 3*B*). Moreover, ERG and TMPRSS2 expressions were also markedly decreased after castration and returned to precastration levels in the relapsed tumors, consistent with AR

reactivation (Fig. 3*B*). Although the importance of ERG expression *in vitro* remains unclear, previous data suggest that ERG functions *in vivo* by inducing genes that enhance tumor invasion, including matrix metalloproteinases (MMP) that are established *Ets* target genes (15–17). Significantly, MMP3 expression markedly declined after castration and returned to at least precastration levels in the relapsed tumors, supporting a role for ERG in recurrence.

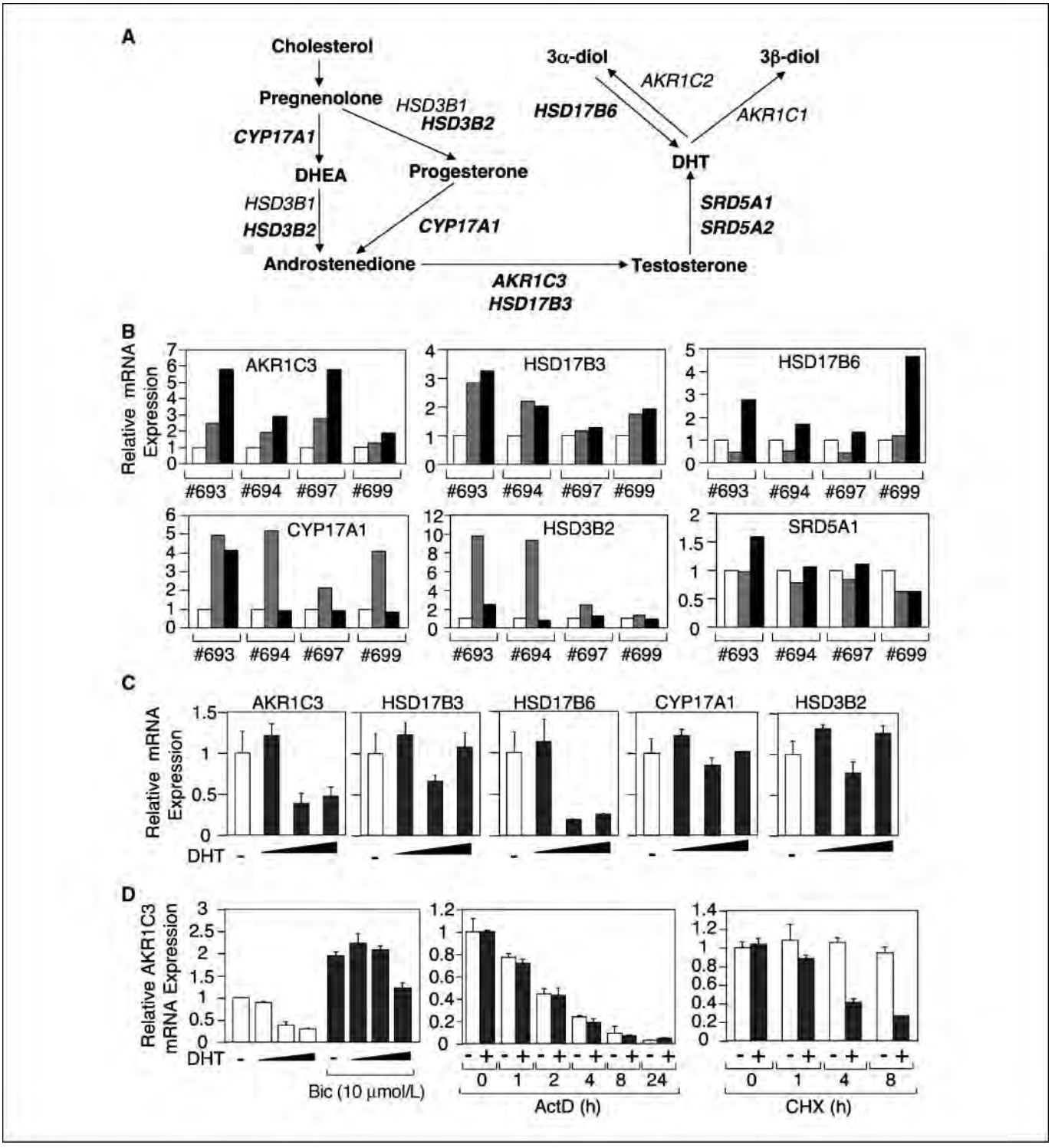

Figure 4. Androgen synthetic enzymes in VCaP xenografts. *A,* enzymes in androgen metabolism. *B,* relative expression of indicated enzymes by RT-PCR in VCaP xenografts (before castration, *blank columns*; 4 d after castration, *gray columns*; relapse, *black columns*). *C,* VCaP cells in CSS medium were treated with 0, 0.1, 1, or 10 nmol/L of DHT for 24 h and assessed by RT-PCR for the indicated transcripts. *D,* VCaP cells grown in CSS medium were treated with (*left*) 0, 0.1, 1, or 10 nmol/L of DHT minus or plus bicalutamide (10 μmol/L) for 24 h, (*middle*) actinomycin D (*ActD*; 10 μmol/L) minus or plus DHT (10 nmol/L) for 0 to 24 h, or (*right*) cycloheximide (*CHX*; 10 ng/mL) minus or plus DHT (10 nmol/L) for 0 to 8 h. Actinomycin D and cycloheximide were added 1 h before DHT. Results were normalized to 18S RNA.

Previous analyses of CRPC clinical samples and xenograft models have shown that AR mRNA is highly expressed and increased compared with levels before androgen deprivation (7, 8, 18, 19). Consistent with these data, AR mRNA in VCaP xenografts was increased after castration and was further increased in the relapsed tumors (Fig. 3*B*). In contrast, AR protein was markedly decreased at 4 days but was increased in the relapsed tumors (Fig. 3*C*). Nuclear ERG and AR expression were observed by immunohistochemistry in the VCaP xenografts before castration (Fig. 3*D*). Both declined markedly 4 days after castration, and the remaining AR at this time seemed to be cytoplasmic. Consistent with the RT-PCR and immunoblotting results, immunohistochemistry showed that both ERG and AR expressions were restored in the relapsed tumors. Whereas AR expression was primarily nuclear before castration, intense nuclear and diffuse cytoplasmic AR expressions were observed in the relapsed tumors. Taken together, these data show that *TMPRSS2:ERG* expression is markedly decreased in response to castration and that expression is restored in conjunction with AR reactivation in relapsed tumors.

Relapsed VCaP xenografts have increased expression of enzymes mediating androgen synthesis. PC may adapt to ADT by enhancing synthesis of androgens from weak steroid precursors and/or by *de novo* synthesis (8, 11, 12). Therefore, we assessed androgen synthetic enzymes before and after castration (Fig. 4*A*). AKR1C3 was increased after 4 days and went up further in the relapsed tumors (Fig. 4*B*). AKR1C3 expression in VCaP cells *in vitro* was also rapidly decreased by DHT, indicating that it is negatively regulated by androgen (Fig. 4*C*). Further experiments showed that this negative regulation was agonist dependent (blocked by an AR antagonist, bicalutamide) and reflected decreased transcription rather than increased mRNA degradation as it was not observed in actinomycin D–treated cells (Fig. 4*D*). Finally, DHT decreased AKR1C3 mRNA in cells treated with cycloheximide, indicating that new protein synthesis was not required and suggesting that the agonist liganded AR may be directly suppressing AKR1C3 transcription.

HSD17B3 in testes carries out the same reaction as AKR1C3 but not normally in prostate. Although it was increased by castration, its basal expression was at the lower limits of detection. HSD17B6, which regenerates DHT from its major metabolite (3α-diol; ref. 20), was also increased in the relapsed tumors (Fig. 4*B*) and was rapidly down-regulated by DHT *in vitro* (Fig. 4*C*). CYP17A1 and HSDB2 were increased after castration but returned to approximately baseline levels in the relapsed tumors (it should be noted that rodent adrenal glands do not express CYP17, so serum levels of "adrenal androgens" are low in castrate mice). These observations indicate that the entire steroidogenesis pathway is up-regulated acutely in response to ADT in VCaP xenografts and that further up-regulation of AKR1C3 and HSD17B6, in conjunction with increased AR, may eventually provide the tumors with adequate androgen for AR reactivation and expression of AR-regulated genes, including *TMPRSS2:ERG*.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Phase II study of androgen synthesis inhibition with ketoconazole, hydrocortisone and dutasteride (KHAD) in asymptomatic castration resistant prostate cancer

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Running title: Combination ketoconazole and dutasteride for CRPC

Key words: prostate cancer, hormonal therapy, ketoconazole, dutasteride, androgen

Statement of Translational Relevance

Androgen receptor (AR) is reactivated in prostate cancer that relapses after androgen deprivation therapy (castration resistant prostate cancer, CRPC), with one mechanism being increased expression of enzymes (AKR1C3 and SRD5A1, respectively) mediating the synthesis of testosterone and dihydrotestosterone (DHT) from precursor steroids. Consistent with this mechanism, inhibitors of CYP17A1 (ketoconazole or the more potent abiraterone), the enzyme that mediates androgen precursor synthesis, are active in CRPC, but responses are partial and transient. In this exploratory single arm phase II study we assessed the efficacy of blocking two steps in androgen synthesis by combining a dual SRD5A1/SRD5A2 inhibitor (dutasteride) with a CYP17A1 inhibitor (ketoconazole) in CRPC. The response rate and marked prolongation in time to progression compared to previous singe agent trials indicate that intratumoral conversion of low levels of testosterone to DHT is a mechanism of resistance to CYP17A1 inhibitors, and support further combination therapy trials.

Abstract

Purpose: Increasing evidence indicates that enhanced intratumoral androgen synthesis contributes to prostate cancer progression after androgen deprivation therapy. This phase II study was designed to assess responses to blocking multiple steps in androgen synthesis with inhibitors of CYP17A1 (ketoconazole) and type I and II 5α-reductases (dutasteride) in patients with castration resistant prostate cancer (CRPC).

Experimental Design: Fifty seven men with CRPC were continued on gonadal suppression and treated with ketoconazole (400 mg TID), hydrocortisone (30 mg/AM, 10 mg/PM) and dutasteride (0.5 mg/day).

Results: PSA response rate (≥50% decline) was 56% (32/57; 95% CI 42.4% to 69.3%). In patients with measurable disease, 6/20 (30%) responded by RECIST criteria. Median duration of treatment was 8 months. In the 25 patients who met criteria for disease progression, median time to progression was 14.5 months. Median duration of response was 20 months; 9 patients remain on therapy with treatment durations censored at 18 to 32 months. DHEA-S declined by 89%, androstenedione by 56%, testosterone by 66%, and DHT declined to below detectable levels, compared to baseline levels with testicular suppression alone. Median baseline levels and declines in DHEA-S, androstenedione, testosterone, and DHT were not statistically different in the responders versus nonresponders, and hormone levels were not significantly increased from nadir levels at relapse.

Conclusion: The response proportion to KHAD was at least comparable to previous studies of ketoconazole alone, while time to progression was substantially longer. Combination therapies targeting multiple steps in androgen synthesis warrant further investigation.

Introduction

Prostate cancer (PC) that progresses after androgen deprivation therapy (ADT), termed castration resistant prostate cancer (CRPC), expresses androgen receptor (AR) and multiple androgen regulated genes at high levels (including *PSA* and *TMPRSS2:ERG* fusion genes), indicating that AR transcriptional activity has been reactivated despite castrate serum androgens levels (1-3). Mechanisms that may contribute to this AR reactivation include increased AR expression (increased AR mRNA in most patients and AR gene amplification in ~30%) (4), AR mutations (primarily in patients treated with an AR antagonist) (5, 6), increased activity of transcriptional coactivator proteins (7, 8), and stimulation of kinases that directly or indirectly enhance AR responses to low androgen levels (9-12).

A further mechanism contributing to tumor progression after ADT is increased intratumoral androgen synthesis. CRPC tumors have increased expression of enzymes mediating testosterone and dihydrotestosterone (DHT) synthesis from weak adrenal androgens (dehydroepiandrosterone, DHEA and androstenedione), and may also upregulate enzymes including CYP17A1 that are required for *de novo* steroid synthesis (3, 13, 14). Consistent with increased intratumoral androgen synthesis in CRPC, androgen levels in the prostates of men who recur locally after ADT are comparable to

levels in the prostates of eugonadal men (15-17). Moreover, testosterone levels in metastatic CRPC samples are actually higher than in prostate prior to castration (13). Significantly, high intratumoral androgen levels, in addition to reactivating AR, may render tumor cells relatively resistant to available weak competitive AR antagonists and contribute to the modest efficacy of these antagonists when used initially in combination with castration (combined androgen blockade) (18) or as secondary hormonal therapy in CRPC (19, 20).

The contribution of androgens produced by the adrenal glands to CRPC was suggested in early adrenalectomy studies (21). Ketoconazole, which inhibits a number of cytochrome P450 enzymes including CYP17A1 that is required for adrenal androgen synthesis, has reported response rates in CRPC ranging from 20-75% (22). The response rate in the largest study of ketoconazole/hydrocortisone (with simultaneous or sequential anti-androgen withdrawal) was 32% when used after anti-androgen withdrawal (23). Interestingly, this study found a positive correlation between responses and pre-therapy levels of androstenedione, and the mean adrenal androgen levels were partially restored at disease progression (24). These observations suggest that responses might be improved with more effective androgen synthesis inhibitors, and encouraging response rates (>50%) have been reported in phase I/II clinical trials of a more potent CYP17A1 inhibitor, abiraterone (22, 25).

While CYP17A1 inhibitors can markedly reduce levels of circulating and presumably intratumoral androgen precursors, tumor cells may still convert any available androstenedione to testosterone (mediated by the enzyme AKR1C3) and then to the higher affinity ligand DHT (mediated by type 1 or type 2 5α-reductases). In normal

prostate, testosterone is reduced to DHT primarily by the type 2 5α-reductase (SRD5A2). Selective inhibition of type 2 5α-reductase with finasteride can decrease PC incidence, but this drug does not have clear activity in CRPC (26). Studies from several groups have shown that expression of the type 1 5α-reductase (SRD5A1) is increased in primary PC, and we found that SRD5A1 expression was further increased in CRPC (3, 27). A dual inhibitor of type 1 and type 2 5α-reductases, dutasteride, is an effective treatment for benign prostatic hyperplasia (BPH) and is under investigation for PC prevention, but its efficacy in CRPC has not been determined (28).

Based on these observations and available drugs, we hypothesized that combined inhibition of CYP17A1 with ketoconazole and inhibition of type 1 and 2 5α-reductases with dutasteride would be an effective therapy in CRPC. We present here results of a phase II trial of combined treatment with ketoconazole, hydrocortisone and dutasteride (KHAD) in CRPC. The trial was designed as an exploratory study to determine whether addition of dutasteride to standard ketoconazole/hydrocortisone would improve responses to at least 50% from the 32% response in the CALGB 9583 study (23), and would be therefore worthy of further study. Additional endpoints included time to progression and correlations between androgen levels and responses.

Materials and Methods

Patients and Eligibility

This open-label single-arm multicenter phase II study was initiated at the Dana Farber/Harvard Cancer Center (DF/HCC) (Beth Israel Deaconess Medical Center, BIDMC and Dana-Farber Cancer Institute, DFCI) and conducted through the

Department of Defense Prostate Cancer Clinical Trials Consortium (trial registration number NCT00673127). From June 2005 through April 2007, 57 patients were consented and started on therapy. Participating institutions included DF/HCC (n=26), Sunnybrook Health Science Centre (n=10), Oregon Health Science University (n=8), MD Anderson Cancer Center (n=8), and Johns Hopkins University (n=5). The institutional review board of each institution approved the trial.

Eligibility included progressive CRPC, defined as a PSA increase over baseline of >25% or 5 ng/ml, or new lesions on bone/CT scan after conventional androgen deprivation and anti-androgen withdrawal. Metastatic disease was not required. Additional criteria included ongoing gonadal androgen ablation with serum testosterone <0.5 ng/ml, PSA $\geq$2 ng/ml, no prior therapy with ketoconazole or corticosteroids for PC, and ECOG performance status of 0-2. Prior chemotherapy was allowed. Patients taking drugs that may prolong QT intervals or known to be narrow therapeutic index CYP3A4 substrates were excluded.

The treatment was ketoconazole 400 mg po TID, hydrocortisone (30 mg in AM and 10 mg in PM) and dutasteride (0.5 mg per day). Dose modifications for toxicity were specified. Patients were evaluated every four weeks, with history, physical exam, and laboratory analysis including liver function tests and PSA. Serum for hormone measurements was obtained every 4 weeks for the first 12 weeks and then every 12 weeks until progression (measured in duplicate by RIA, Diagnostic Systems Laboratories, Webster, TX). Measurable disease was evaluated by CT and bone metastasis by bone scan every 12 weeks. Toxicity was graded according to the NCI CTC v3.0.

Endpoints

PSA response was defined as a decline of at least 50% from baseline confirmed by a second measurement at least 4 weeks later; the reference for these declines was measured within 2 weeks prior to starting therapy. PSA progression was defined according to PSA Working Group criteria (29). Measurable disease response and progression were evaluated according to RECIST criteria. Progressive non-measurable disease was defined as 2 or more new lesions on bone scan, appearance of new non-bony metastases, or development of an indication for radiation therapy. Time to progression (TTP) was defined from the date of treatment initiation until the date that PSA progression criteria were first met or the date of measurable or non-measurable disease progression; otherwise it was censored at the date of the last PSA measurement without evidence of disease progression. Among patients who achieved a ≥50% PSA decline, PSA response duration was defined from the date of the first 50% PSA decline until the date of PSA or disease progression, or was censored at the date of the last PSA measurement without progression. Hormone levels were compared using Wilcoxon rank sum tests.

Statistical Considerations

Enrollment proceeded in a two-stage design to differentiate a response rate of $\geq$50% from a response rate of $\leq$32% with types I and II error of 0.10. Upon assessment that $\geq$8 of the initial 23 patients had responded, enrollment continued to a total of 57 patients. The PSA response rate and exact binomial 95% CI are reported. All patients,

regardless of their disease evaluations, are included in the assessment of response. The PSA response duration and TTP distributions were summarized using the Kaplan-Meier method with 95% CI.

Results

Patient Characteristics

Patient and disease characteristics are in Table 1. The mean age at enrollment was 68 years, and the median duration of primary ADT was 3.4 years. Median baseline PSA was 24 ng/ml (range, 3.7 -2,740 ng/ml), and median PSA doubling time prior to entry was 2.4 months. Measurable disease was present in 35% of patients, and 70% had bone metastases. Prior treatments included bicalutamide in 89% of patients, and chemotherapy in 5% of patients.

Treatment Activity

The PSA response rate (confirmed ≥50% decline in PSA from baseline) was 56% (32/57; 95% CI 42.4% to 69.3%). PSA declined by ≥80% in 27/57 patients (47%) and by ≥90% in 16/57 patients (28%) (Figure 1). Among the 32 PSA responders, the median PSA response duration was 20 months (95% CI 13.5 to undetermined upper limit); 9 patients were continuing on treatment without progression at the time of this report with treatment durations censored at 18 to 32 months. PSA response rates according to type of disease at baseline (measurable, non-measurable, PSA only) are shown in Table 2, and were similar in all groups.

Amongst the 20 patients with measurable disease at baseline, 6 (30%) had complete or partial responses by RECIST criteria, 5 (25%) had stable disease, and 1 had progressive disease. The remaining 8 patients discontinued participation in the study due to PSA progression (3 patients) or for other reasons (5 patients) before measurable disease responses could be assessed. The one patient with a CR had baseline PSA of 2740 ng/ml, a 1.5 cm lymph node metastasis, and bone metastases. The target lymph node metastasis was not present on 6, 9, 12 or 15 month CT scans and nadir PSA was 2.1 ng/ml at 8 months. This patient stopped treatment after ~15 months by choice with a rising PSA of 6 ng/ml.

There were 40 patients with bone metastases at baseline. Eighteen (40%) had stable disease (0 or 1 new lesions on bone scan) throughout the therapy, and 15 of these 18 had PSA responses. One patient had stable bone metastases for >1 year, but then had bone progression resulting in treatment cessation. Eight patients had progression of bone metastases at the first bone scan (2 of these 8 patients had PSA responses within those first 3 months of treatment). The remaining 13 patients came off treatment within 3 months and did not have a follow-up bone scan (3 of these 13 had PSA progression).

The median time to disease progression was 14.5 months (95% CI, 11.0 to upper limit undetermined; Figure 2). The median duration of treatment was 8 months, ranging from <1 month to >32 months. Forty percent (22/57) continued treatment for $\geq$1 year. Ten patients stopped treatment with the reason specified as toxicity or side effects. An additional 10 patients stopped treatment by physician discretion or patient choice. One

patient stopped treatment at 5 weeks for pain management, and 1 patient stopped at 8 months for nerve root compression.

Toxicity

Toxicities were reported among all patients, and 42% (24/57) of patients experienced at least one grade 3 to 4 toxicity. In 6 of these, the only reported grade 3 toxicity was impotence. Grade 3 hypertension occurred in 4 patients, hyperglycemia in 3 patients, hypokalemia in 2 patients, and elevated ALT/SGPT in 2 patients. Grade 3 lymphopenia, rash, hot flashes, nausea, bladder hemorrhage, infection, AST/SGOT, creatinine, nonneuropathic generalized weakness, cognitive disturbance, pain, dyspnea, urinary frequency/urgency were reported each for one patient. There was one grade 4 thrombosis.

Hormone Data

Baseline serum samples were available from 41 patients, and there was at least one subsequent on-therapy serum sample from 33 of these 41 patients. Median DHEA-S at baseline was 599 ng/ml and fell to 65 ng/ml after ~1 month on therapy (median 89% decline) (Table 3). Baseline median androstenedione was 0.84 ng/ml and dropped by a median of 56% to 0.22 ng/ml at one month. Baseline median testosterone was 0.37 ng/ml and at one month there was a 66% drop to a median of 0.13 ng/ml. Baseline median DHT was 2.6 pg/ml. This declined after 1 month in all patients, with the median at 1 month being below the level of sensitivity (<2.0 pg/ml). There was no further significant decline in any of these hormones after 2-3 months of therapy.

Baseline median levels of androstenedione, testosterone and DHT were similar in the patients who responded to therapy (N=20) versus the nonresponders (N=21) (Table 4). Baseline DHEA-S was higher in the responders (840 ng/ml) versus the nonresponders (480 ng/ml), but this was not statistically significant. Median decline in DHEA-S after 1 month of therapy was also greater in the responders (93%) versus the nonresponders (85%), but this difference did not reach significance (Table 4). The declines in androstenedione, testosterone, and DHT were comparable in the responders versus nonresponders. Amongst the responding patients who had progressed at the time of this analysis, paired serum samples after one month on therapy and at the time of progression were available from 15 cases. There was no significant increase in the median ratio at progression versus 1 month for DHEA-S (1.17 CI 0.71, 1.92) , androstenedione (1.11 CI 0.65, 1.92), testosterone (1.34 CI 0.89, 1.99), or DHT (1.11 CI 0.66, 1.82).

Discussion

We conducted an exploratory phase II study to assess the efficacy of treatment with ketoconazole/hydrocortisone in combination with dutasteride in men with CRPC.
The largest previous study of ketoconazole (CALGB 9583) analyzed responses to ketoconazole/hydrocortisone given concurrently or subsequent to antiandrogen withdrawal (response rates of 27% and 32%, respectively), so the current study was powered to determine whether the response was greater than the rate of ~32% in CALGB 9583 (23). Although results in a single-arm study must be viewed with caution, and response rates to ketoconazole in other smaller studies have ranged from 20-75%

(23), the 56% response rate to KHAD indicates that 5α-reductase inhibition by dutasteride may enhance the response rate to ketoconazole/hydrocortisone.

Importantly, while comparable response rates have been reported for ketoconazole alone, response durations in this study were markedly longer than those in previously reported studies of ketoconazole/hydrocortisone. The median duration of response was 20 months, and 9 of the 32 responding patients had not yet progressed at the time of this analysis (at durations of 18-32 months). Among all patients, median TTP was 14.5 months. This is markedly longer than the median time to PSA progression of 8.6 months reported in CALGB 9583 (23), and longer than the median response durations of between 3.3 to 9 months in seven other reported ketoconazole trials (22). It is also longer than the median TTP of 7.5 months in phase II studies of abiraterone (see below) (30).

As expected, KHAD caused a median decline of ~90% in serum DHEA-S levels, and declines of ~50-70% in serum androstenedione and testosterone. These declines in DHEA-S and androstenedione are comparable to those in CALGB 9583 (95% and 58%, respectively), although median testosterone did not markedly decline in the CALGB study (0.13 to 0.11 ng/ml). Baseline median levels of androstenedione and testosterone were comparable amongst responders and nonresponders, while median DHEA-S was higher in the responders (840 versus 480 ng/ml respectively), although this difference was not statistically significant. Interestingly, higher baseline androstenedione and DHEA-S levels were correlated with responses to ketoconazole/hydrocortisone plus antiandrogen withdrawal in the CALGB 9583 study, although only the androstenedione association was significant. A further finding in CALGB 9583 was that median levels of

DHEA-S and androstenedione, which had declined after 1 month of therapy, were increased relative to nadir levels at the time of progression. We did not observe a consistent increase in any of the measured hormones at relapse, but samples from patients who are still responding remain to be examined.

DHT also declined in all patients, but basal levels were already at the limit of sensitivity and the median level after 1 month was below the sensitivity of the assay. Previous studies have not examined the effects of ketoconazole/hydrocortisone alone on DHT in castrate men, so we cannot determine the extent to which DHT levels were further suppressed by dutasteride. More importantly, concentrations of DHT (and of testosterone) in the tumor are likely higher than serum levels, and may more closely correlate with responses (13, 16, 17). Measurement of intratumoral androgen levels in response to therapy is clearly a major challenge, but should be an objective in future studies.

Abiraterone is a more potent and specific CYP17A1 inhibitor than ketoconazole, with phase I studies showing >95% declines in serum testosterone to <0.01 ng/ml (25). Nonetheless, despite this more marked decrease in serum testosterone levels, the reported response rate to abiraterone in phase I/II studies (>50% PSA decline in 67%, >90% PSA decline in 19%) appears comparable to the KHAD response rate (25, 30). Moreover, as noted above, median time to PSA progression on abiraterone was 7.5 months versus 14.5 months for KHAD. These observations suggest that intratumoral conversion of weak androgens to testosterone and DHT may still be contributing to treatment resistance and relapse in abiraterone treated patients. In contrast, the addition of dutasteride to ketoconazole, by blocking intratumoral conversion of

testosterone to DHT, may result in extremely low intratumoral DHT levels that compensate for the somewhat higher levels of residual testosterone. It also should be noted that dutasteride will presumably increase the aromatization of testosterone to estradiol and decrease the levels of DHT metabolites that may be estrogen receptor β ligands, although the net effect of this altered testosterone metabolism on tumor growth is uncertain. In any case, the hypothesis that intratumoral DHT synthesis contributes to abiraterone resistance should be tested in a randomized phase II trial.

Early studies employed adrenalectomy or hypophysectomy as therapies for CRPC, with about one-third of patients having objective responses in measurable disease and the majority having subjective improvement (21). Adrenalectomy clearly ablates adrenal androgen synthesis, and these responses may reflect the maximal effects one can hope to obtain by inhibiting androgen synthesis in CRPC. However, there are likely other sources of weak androgens, including CRPC cells that may express increased levels of CYP17A1, although it remains to be established whether CRPC cells synthesize substantial levels of androgens *de novo* from cholesterol (13, 14). In any case, we suggest that adrenalectomy does not reflect maximal androgen deprivation, and that androgen levels (particularly intratumoral levels) may be further reduced by more potent inhibitors of androgen synthesis, or by combinations of inhibitors that block at multiple steps.

In summary, this study indicates that dutasteride may enhance the response rate to ketoconazole/hydrocortisone, and substantially increases the duration of response and overall TTP. This result supports the conclusion that DHT synthesis by the type 1 5α-reductase, which is increased in CRPC, contributes to PC survival and progression

after ADT. Further randomized trials of 5α-reductase inhibitors in conjunction with ketoconazole or more potent CYP17A1 inhibitors are warranted. Moreover, further efforts should be made to develop inhibitors of other enzymes mediating androgen synthesis. Finally, it will clearly be important to determine whether these efforts to more effectively suppress androgen synthesis and AR activity lead to improved survival.

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Table 1. Patient and disease characteristics

Characteristic	Number (%) of Patients or Median [range] of values
Number of patients	57(100)
Non-Caucasian	5 (9)
Hispanic or Latino	1 (2)
At Initial PC Diagnosis	
Age (yrs)	61 [44 – 80]
PSA (ng/mL), n=46	16 [3 – 1549]
Gleason Score	
4-6	14 (25)
7	16 (28)
8-9	20 (35)
Unknown	7 (14)
Prior Treatments	
Duration of primary ADT (yr), n=54	3.4 [0.2 – 15.5]
LHRH agonist	51 (89)
Orchiectomy	4 (7)
Anti-androgens (any)	52 (91)
Bicalutamide	51 (89)
Nilutamide	6 (11)
Flutamide	5 (9)
Finasteride	2 (4)
Estrogens (including estramustine)	8 (14)
Chemotherapy	3 (5)
At Registration	
Age (yr)	68 [48 – 90]
Years since diagnosis	6.6 [0.3 – 20]
ECOG PS 1	16 (28)
PSA (ng/mL)	24 [3.7 – 2740]
PSA doubling time (mo), n=51	2.4 [1 – 24]
Alkaline phosphatase (u/L)	84 [30 – 527]
Hemoglobin (g/dL)	13.1 [10.7-15.6]
Type of disease	
Measurable disease	20 (35)
Measurable only	6 (11)
Measurable and non-measurable	14 (25)
Non-measurable disease only	28 (49)
PSA only	9 (16)
Sites of disease (multiple)	
Viscera	6 (11)
Soft tissue	6 (11)
Lymph nodes	23 (40)
Bone	40 (70)

Table 2. PSA response rates by type of disease at baseline

	N Enrolled	N (%) PSA Response
Measurable disease ± non-measurable disease	20	11 (55)
Measurable only	6	3 (50)
Measurable and non-measurable	14	8 (57)
Non-measurable only	28	14 (50)
PSA only	9	7 (78)
Overall	57	32 (56)

Table 3. Hormone levels over time among all patients

	Baseline	Month 1		Month 2		Month 3	
Hormone	Value	Value	Percent change from baseline	Value	Percent change from baseline	Value	Percent change from baseline
N patients	N=41	N=33	N=30	N=28	N=24	N=28	N=24
Androstenedione (ng/ml)	0.84 (0.61, 1.10)	0.22 (0.20, 0.36)	-56% (-75%, -43%)	0.27 (0.20, 0.32)	-68% (-74%, -49%)	0.25 (0.20, 0.31)	-58% (-77%, -49%)
DHEA-S (ng/ml)	599 (340, 121)	65 (36, 125)	-89% (-94%, -70%)	65 (37, 106)	-92% (-94%, -77%)	65 (35, 100)	-90% (-94%, -81%)
Testosterone (ng/ml)	0.37 (0.26, 0.47)	0.13 (0.10, 0.15)	-66% (-72%, -55%)	0.11 (0.9, 0.14)	-68% (-73%, -54%)	0.12 (0.10, 0.19)	-64% (-72%, -45%)
DHT[1] (pg/ml)	2.6 (<2.0, 3.8)	<2.0 (<2.0, <2.0)					

Values are median and interquartile range (25^{th}, 75^{th} percentiles).

[1]Note: For DHT, n=33 at baseline, n=32 at month 1 and n=29 for percent change.

Table 4. Hormone levels at baseline and percent change at month 1, according to PSA response at 3 months.

	Baseline			Percent change from baseline to month 1		
Hormone	PSA Responders	PSA non-responders	P-value*	PSA Responders	PSA non-responders	P-value*
N patients	N=20	N=21		N=14	N=16	
Androstenedione (ng/ml)	0.82 (0.6, 1.18)	0.85 (0.63, 0.98)	0.98	-58% (-73%, -43%)	-56% (-77%, -45%)	0.82
DHEA-S (ng/ml)	840 (397, 1145)	480 (311, 1491)	0.56	-93% (-95%, -85%)	-85% (-91%, -61%)	0.10
Testosterone (ng/ml)	0.33 (0.26, 0.50)	0.39 (0.23, 0.47)	0.91	-66% (-74%, -56%)	-61% (-70%, -49%)	0.29
DHT[1] (pg/ml)	2.3 (<2.0, 3.7)	3.0 (<2.0, 4.0)	0.63			

Values are median and interquartile range (25^{th}, 75^{th} percentile).
*P-value by Wilcoxon rank sum test comparing PSA responders vs. PSA non-responders.
[1]Note for DHT, at baseline N=16 and N=17 for PSA responders and non-responders, and N=16 and N=15 respectively for percent change from baseline to month 1.

FIGURE LEGENDS

Figure 1. Maximal change in PSA from baseline among 57 CRPC patients treated with KHAD. Y-axis is truncated at +100%, but 3 patients had 100% or greater increase in PSA from baseline without decline. In total 9 patients did not have any decline in PSA.

Figure 2. Time to progression among 57 CRPC patients treated with KHAD.

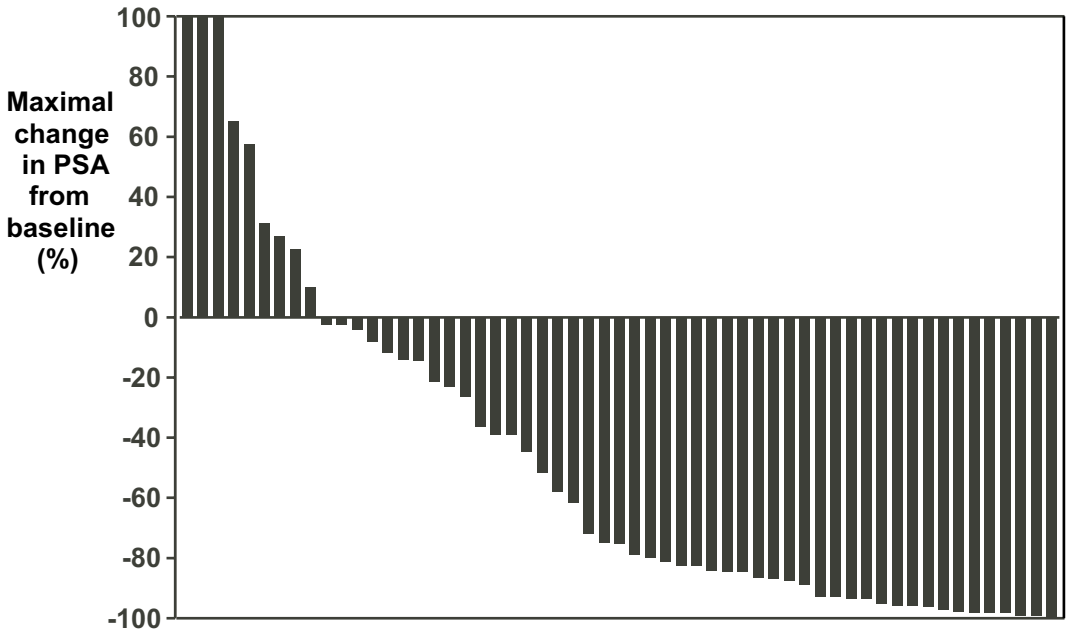
Maximal change in PSA from baseline (%)
100
80
60
40
20
0
-20
-40
-60
-80
-100

Figure 1

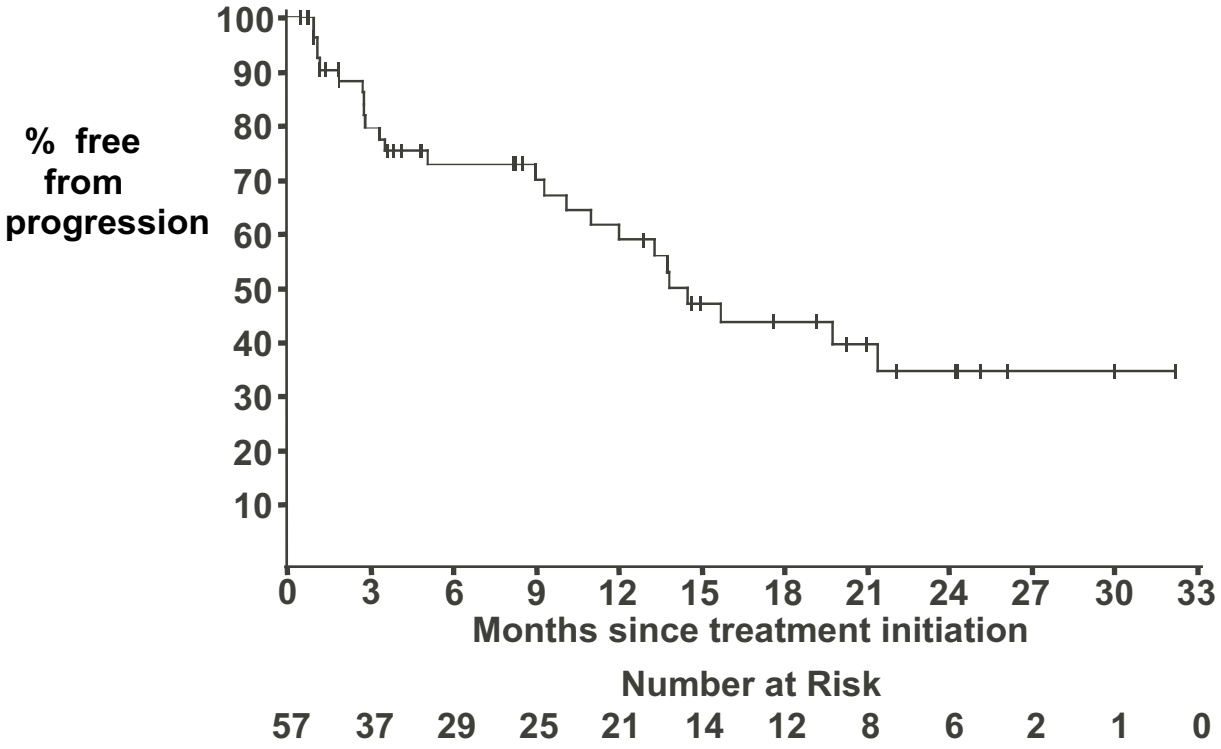
% free from progression
100
90
80
70
60
50
40
30
20
10
0
3
6
9
12
15
18
21
24
27
30
33
Months since treatment initiation
Number at Risk
57
37
29
25
21
14
12
8
6
2
1
0

Figure 2